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The potential for targeted rewriting of epigenetic marks in COPD as a new therapeutic approach

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an age and smoking related progressive, pulmonary disorder presenting with poorly reversible airflow limitation as a result of chronic bronchitis and emphysema. The prevalence, disease burden for the individual, and mortality of COPD continues to increase, whereas no effective treatment strategies are available. For many years now, a combination of bronchodilators and anti-inflammatory corticosteroids has been most widely used for therapeutic management of patients with persistent COPD. However, this approach has had disappointing results as a large number of COPD patients are corticosteroid resistant. In patients with COPD, there is emerging evidence showing aberrant expression of epigenetic marks such as DNA methylation, histone modifications and microRNAs in blood, sputum and lung tissue. Therefore, novel therapeutic approaches may exist using epigenetic therapy. This review aims to describe and summarize current knowledge of aberrant expression of epigenetic marks in COPD. In addition, tools available for restoration of epigenetic marks are described, as well as delivery mechanisms of epigenetic editors to cells. Targeting epigenetic marks might be a very promising tool for treatment and lung regeneration in COPD in the future.

1. Introduction

Epigenetics is generally defined as the study of heritable or acquired mitotically stable changes in gene expression that occur without variation in DNA sequence. Epigenetic marks can be mainly subdivided in three classes: DNA methylation, post-translational histone modifications (histone PTMs, including histone acetylation, methylation, phosphorylation, ubiquitination and sumoylation) and non-coding RNAs, with all three probably induced by environmental factors, diet, diseases, and processes involved in ageing (Fraga et al., 2005; Yang & Schwartz, 2011). All somatic cells in an organism initially have the same DNA sequence, but somatic mutations accumulate over time, which contributes to phenotype changes in a variety of diseases including COPD and lung cancers. In recent years, evidence has increased that epigenetic mechanisms influence gene expression in chronic lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD). As such, epigenetics provides the link between genetic factors and environmental exposures (Kraussetschmann, Meyer, Dehmel, & Hylkema, 2015; Yang & Schwartz, 2011).

1.1. DNA methylation

DNA methylation is a process by which methyl groups are added to cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides and catalyzed by DNA methyltransferase (DNMT) family members. The DNA methylation status is associated with the activity of a DNA

Abbreviations: COPD, Chronic obstructive pulmonary disease; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-Associated Protein 9 (Cas9); CSC, Cigarette smoke condensate; DNMT, DNA methyltransferase; EV, Extracellular vesicles; HAT, Histone acetyltransferase; HDAC, Histone deacetylase; IVT-mRNA, *In vitro* transcribed resulting in messenger RNA; iTOP, Induced transduction by osmocytosis and propanebetaine; LNP, Lipid nanoparticles; miRNA, microRNA; PBAE, Poly (β-amino ester); PEI, Polymer polyethyleneimine; PEG, Polyethylene glycol; rAAV, recombinant adeno-associated virus; rAd, recombinant adenovirus; sgRNA, single guide RNA; TALEs, Transcription-Activator-Like Effectors; ZFPs, Zinc finger proteins

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segment and does not affect the sequence. Generally, DNA hypermethylation of CpG islands in gene promoter regions usually leads to gene silencing, and hypomethylation triggers transcription activation (Yang & Schwartz, 2011).

1.2. Post-translational histone modifications

Chromatin is organized into subunits (called nucleosomes), which consist of a protein octamer, including two copies of each core histone H2A, H2B, H3 and H4 where 147 bp DNA is wrapped around with, and the linker H1. Histones are the main protein components of chromatin, playing an important role in gene regulation. The histone PTMs that mainly occur on the histone tails form a histone code which can be deciphered by other proteins. Many studies have established that H3K4me3 is closely associated with the activation of gene expression, while H3K9me3 and H3K27me3 are related to gene repression.

1.3. Non-coding RNAs

Non-coding RNAs (ncRNAs) are classified into four well-characterized forms: microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs) and long non-coding RNAs (LncRNAs) (Jodar, Selvaraju, Sendler, Diamond, & Krawetz, 2013). miRNAs are short non-coding RNA molecules containing approximately 22 nucleotides, that negatively regulate gene expression through interacting with the 3′-UTR (untranslated regions) of mRNA (messenger RNA) which result in the degradation of mRNA or repression of protein translation (Bartel, 2004; Flynt & Lai, 2008). For a long time, the main function of piRNA was thought to be the protection of germ cell genome integrity by silencing mRNA of transposable elements, which can interrupt the genome by insertion or transposition (Siomi, Sato, Pezic, & Aravin, 2011). However, recent studies have elucidated additional roles showing their involvement in genome rearrangement and epigenetic programming (Ross, Weiner, & Lin, 2014).

1.4. COPD and current treatment

COPD is a progressive disease, characterized by irreversible airway obstruction, excessive mucus secretion, inflammation, extracellular matrix destruction and abnormal tissue repair (Celli et al., 2004). According to the World Health Organization (WHO), over 200 million people worldwide suffer from COPD, with the incidence increasing with ageing. The disease is currently the third leading cause of death worldwide (World Health Statistics, 2008). The most important risk factor for developing COPD is cigarette smoking, but other inhaled noxious particles and gases are also recognized for their pathogenic role (Celli et al., 2004).

Currently, there is no cure for COPD. After diagnosis, the goal of clinical treatment of COPD is to improve a patient's health status and quality of life. Aside from smoking cessation being the foremost module for currently smoking patients (Schamberger, Mise, Meiners, & Eickelberg, 2014; Willemse et al., 2005), maintenance therapy with inhaled corticosteroids (IHC) - in combination with long-acting bronchodilators- have formed a cornerstone for management of moderate and severe COPD for almost 20 years. In particular patients with enhanced eosinophilic counts in blood respond well to high doses of the combination of IHC/long-acting β2-agonist (Pavord et al., 2015). However, whereas severe asthma patients with high eosinophilic levels might benefit from anti-eosinophil therapies, including blocking antibodies against IL-5, IL-13 and IL-33 (Barnes, 2015), it yet has to be proven if these immunotherapeutic treatments are beneficial for (a subgroup of) COPD patients as well (Barnes, 2015). Regarding patients with enhanced neutrophilic counts, they seem to benefit from treatments with macrolide antibiotics, which next to their classical antimicrobial actions could also be due to their anti-inflammatory or immunomodulatory effects (Ni et al., 2014; Uzun et al., 2014). Moreover, other sorts of health care including health education on nutrition and exercise programs are also recommended for a subgroup of patients with COPD (Deane et al., 2017). COPD can present with different phenotypes, most likely resulting from various molecular endotypes. Therefore, to further improve treatment of COPD, novel strategies for a more personalized therapeutic approach are urgently required (Heaney & Mcgarvey, 2017). Such approaches could arise from the discovery of new therapeutic targets via genome-wide association studies (GWAS) and epigenetic studies. The dysregulated expression of genes in COPD such as *FAM13A* (Chen et al., 2015; Michael et al., 2010), *CHRNA 3/5*, *IRES2* and *HHIP* (Pillai et al., 2009) were discovered through population-based GWAS, which represent potential target genes for COPD therapy. Until now, the mechanisms altering COPD pathology are mostly unknown and none of the identified genes are specifically targeted by COPD therapeutic approaches yet. Epigenetic marks, different from genetic mutations, underlie heritable yet reversible changes in gene expression, which makes epigenetic marks attractive for targeted therapy. It is hoped that the development of epigenetic technologies will offer an effective clinical therapy for COPD in the near future. In the following, we will summarize current literature about DNA methylation, histone modifications and non-coding RNAs in COPD and discuss potential possibilities for epigenetic rewriting.

2. Epigenetic dysregulation in COPD

2.1. DNA methylation

Cigarette smoking stimulates an inflammatory response and increases the production of reactive oxygen species (ROS) in the lung, which promotes the pathophysiological changes related to COPD and adds to the risk for development of lung cancer (Adcock, Caramori, & Barnes, 2011). Indeed, emerging evidence exists that cigarette smoking is associated with epigenetic changes in blood cells (Breitling, Yang, Korn, Burwinkel, & Brenner, 2011; Qiu et al., 2011; Wan et al., 2012), bronchial epithelium (Belinsky et al., 2002; Buro-Auriemma et al., 2013; Song, Heijink, et al., 2017), lung tissue (Sundar et al., 2017) and sputum cells (Belinsky et al., 2002; Sood et al., 2010). DNA methylation is the epigenetic mark that is analyzed in all epigenome-wide association studies (EWASs) investigating epigenetic dysregulation in smokers, with or without COPD. As high throughput platforms are available that currently enables the interrogation of over 850,000 (850 K) methylation sites quantitatively across the genome at a single-nucleotide resolution. These published EWASs, covering 27 K or 450 K methylation sites, have shown a clear association between smoking and altered DNA methylation patterns for a number of genes, which are mostly cancer-related (Belinsky et al., 2002; Breitling et al., 2011; Monick et al., 2012; Wan et al., 2012; Zeilinger et al., 2013). So far the largest EWAS by Zeilinger, reported in 2013, discovered differences in the methylation of 187 CpG sites between current smokers and never smokers in which, with a few exceptions, active smoking was associated with reduced methylation levels (Zeilinger et al., 2013). Interestingly, smoking-related changes in methylation turned out to be reversible, with intermediate methylation levels among former smokers compared to current and never smokers (Xu, Jia, Zhang, Breitling, & Brenner, 2015). This reversibility of DNA methylation, related to dose and time of smoking exposure, indicates that DNA methylation could be used as a biomarker for COPD (Bojesen, Timpson, Relton, Smith, & Nordestgaard, 2017; Shenker et al., 2013; Zhang, Yang, Burwinkel, Breitling, & Brenner, 2014; Zhang, Yang, Burwinkel, Breitling, Holleczek, et al., 2014). Two recent studies in a Korean COPD cohort confirmed previous findings identified in Caucasians but also discovered novel smoking associated DNA methylation changes in blood (Lee, Hong, Kim, Kim, & London, 2017; Lee, Hong, Kim, London, & Kim, 2016). These methylation changes correlated with smoking intensity and history. Furthermore, differential methylation

could be associated with expression profiles of corresponding genes in COPD lung tissues, supporting a possible epigenetic role in the control of lung function (Lee et al., 2017).

The use of DNA methylation profiles in peripheral blood as a biomarker for risk of disease and response to therapy, is an attractive concept as it could be translated into clinical practice with relative ease. Even more interesting is that identified smoking-related differential methylation signals in peripheral blood were also shown to influence risk for development of COPD and lung function (Lee et al., 2017; Machin et al., 2017; Qiu et al., 2011; Sood et al., 2010; Wielscher et al., 2015). One result of interest would be hypomethylation of CpG site cg02181506 in the *SERPINA1* gene on chromosome 14, which was associated with COPD (Qiu et al., 2011). However, all these reports in peripheral blood were based on cross sectional analyses of DNA methylation and lung function, and there are no consistent findings across the identified studies, possibly due to the heterogeneity of methods used in the various studies (Machin et al., 2017).

In sputum, aberrant promoter methylation of the *p16* or *GATA4* genes was found to be associated with low lung function and increased odds of COPD among smokers (Meek, Sood, Petersen, Belinsky, & Tesfaigzi, 2015; Sood et al., 2010). Furthermore, airway *GATA4* gene methylation status may also independently predict health status in individuals with COPD (Meek et al., 2015). Also, increased promoter methylation of lung cancer-associated genes, such as *SULF2*, was observed in sputum DNA of ex-smokers with persistent chronic mucous hypersecretion (CMH), which is a clinical phenotype of COPD (Bruse et al., 2014).

Interestingly, the aberrant DNA methylation parameters which correlated with COPD also include overexpression of enzymes involved in DNA methylation: DNA methyltransferase 1 (DNMT1), DNMT3a and DNMT3b. DNMT1 is the most abundant DNA methyltransferase in mammalian cells, and is considered to be the key maintenance methyltransferase in mammals (Bayarsaihan, 2011). DNMT3a and DNMT3b are *de novo* DNA methylation enzymes. A few publications are available regarding the correlation between DNMTs and COPD: Liu reported a reduction of DNMT1 and an augmentation of DNMT3b expression in normal human small airway epithelial cells and human bronchial epithelial cells (HBECs) after chronic exposure to cigarette smoke condensate (CSC) (Liu et al., 2010). Besides that, the genes *D4Z4*, *NBL2*, and *LINE-1* repetitive DNA sequences showed a time dependent hypomethylation in HBECs exposed to CSC (Liu et al., 2010). In addition, hypermethylation of *IL-12Rbeta2* and *WIF-1* frequently occurred in the transition of COPD to lung cancer (Suzuki et al., 2010). However, to understand the consequences of aberrant DNA methylation in various cell types, future research must include functional studies investigating (reprogramming of) DNA methylation in specific cell types (Song, Cano-Rodriquez, et al., 2017; Song, Heijink, et al., 2017), which eventually may lead to the discovery of new targets for COPD therapy.

2.2. Histone modifications

Histone acetylation and histone deacetylation are critical regulators of gene transcription (Marwick et al., 2005; Zong, Ouyang, & Chen, 2015). The imbalance of histone acetylation and deacetylation, modify nucleosomal structure in the transcription of inflammatory cytokine genes, may thus lead to altered gene expression profiles in smokers susceptible to the development of COPD (Szulakowski et al., 2006).

Some years ago, the role of HAT subtypes in the pathology of cancer, asthma and COPD was suggested (Barnes, Adcock, & Ito, 2005; Dekker & Haisma, 2009), and it was proposed that specific inhibitors of these HAT subtypes (p300, CBP, PCAF and GCN5) are potential targets for pharmacological research and clinical applications (Dekker & Haisma, 2009). In addition, histone deacetylase (HDAC) activity and expression, especially for HDAC2, was also shown to be decreased in specimens of COPD patients (Adenuga, Yao, March, Seagrave, & Rahman, 2009; Chen et al., 2012; Ito et al., 2001; Ito et al., 2005). This is of interest as the reduction of HDAC2 activity in patients with COPD leads to expansion of inflammation and corticosteroid resistance (Barnes, 2005). Recently, the effect of the HDAC1-3-selective inhibitor MS-275 was investigated in a cigarette smoke model in C57Bl/6 mice. In this model, where mice were exposed to cigarette smoke for 4 consecutive days, MS-275 robustly attenuated expression of inflammatory cytokines KC (mouse IL-8), IL-6 and IL-1 beta and neutrophil influx in the lungs (Leus et al., 2017). The release of pro-inflammatory cytokines relies on the activity of the transcription factor nuclear factor kappa B (NF-κB), which cooperates with co-activators, such as p300 and CREB-binding protein (CBP), containing histone acetyltransferase (HAT) activity, to activate gene transcription (Adcock, Tsaprouni, Bhavsar, & Ito, 2007; Barnes et al., 2005). The observed anti-inflammatory effect of HDAC inhibitor MS-275 in smoke-exposed mice was partly attributed to the anti-inflammatory cytokine IL-10, which was highly upregulated in MS-275 treated macrophages (Leus et al., 2017). In another study, Clarke et al. reported that protein kinase C (PKC) beta increased TNFα-induced NF-κB transcriptional activity at the promoter of *CCL11* through recruiting p300/CBP-associated factor and acetylation of histone H4 in human airway smooth muscle cells (Clarke et al., 2008).

Aside from acetylase and deacetylase, histone methyltransferase (HMT) and histone demethylase (HDM) play important roles in regulating histone methylation. Compared with histone acetylation, histone methylation is a little more complicated: methylation of histone lysine or arginine residues results in distinct and sometimes even opposite functional outcomes. In addition, the same lysine residues may have different intensity of methylation (mono-, di- or tri-methylation), which lead to varied functions (Mosammaparast & Shi, 2010). For example, Histone H3 lysine 4 tri-methylation (H3K4me3) is generally found on active genes while methylations of H3K9 and H3K27 are closely related to gene repression. Until now, research focusing on the mechanism of histone methylation in COPD is limited. Andresen et al. found that the extent of H3K4me3 significantly associates with increasing beta-Defensin 1 (*DEFB1*) mRNA levels, which closely correlates with pathological progression for COPD (Andresen, Günther, Bullwinkel, Lange, & Heine, 2011). Moreover, Yildirim et al. demonstrated that the mRNA and protein levels of PRMT2, a protein arginine methyltransferase, were upregulated in mouse lung tissue when exposed to hypoxia, which is a latent stimulus for COPD (Yildirim et al., 2006). Overall, histone methylation may be involved in the pathogenesis of COPD, and further studies are needed to expose the underlying mechanisms.

2.3. microRNA

MicroRNA (miRNA) are, so far, the most characterized and well known family of non-coding RNAs. These are characterized as single stranded and 21–23 nucleotides in length. To date, 1881 human miRNAs have been identified according to (miRBase.org, 2017). After formation of the RNA-induced silencing complex (RISC) with Argonaute proteins, miRNAs bind to the 3′ UTR of target mRNAs. Thereby they post-transcriptionally regulate the gene expression by inducing mRNA degradation, destabilization by deadenylation or inhibition of translation (Filipowicz, Bhattacharyya, & Sonenberg, 2008). The possibility of imperfect base pairings (except for the seed region) in miRNA-mRNA interactions allows single miRNAs to regulate numerous targets throughout the genome and thus it is estimated that miRNAs regulate > 60% of all protein-coding genes in humans (Friedman, Farh, Burge, & Bartel, 2009). Further, miRNAs tend to address several key regulatory molecules in signaling pathways, which is why they are often considered as master regulators of gene expression (Chen, Chen, Fuh, Juan, & Huang, 2011; Shalgi, Lieber, Oren, & Pilpel, 2007; Zhou, Ferguson, Chang, & Kluger, 2007). Their expressions ~~is~~ are highly influenced by the environment, and along this line environmental

stressors such as air pollution (Bollati et al., 2010) or cigarette smoke (Marczylo, Amoako, Konje, Gant, & Marczylo, 2012) have been shown to alter miRNA levels. The resulting altered global target gene expression might have detrimental effects on cell homeostasis and could therewith be implicated in the early pathogenesis of various diseases. This is strengthened by the fact that aberrant miRNA expression has been associated with many human pathologies (Poy et al., 2004), including chronic lung diseases such as asthma (Donaldson et al., 2013; Jardim, Dailey, Silbajoris, & Diaz-Sanchez, 2012; Solberg et al., 2012; Williams et al., 2009) or COPD (Akbas, Coskunpinar, Aynacı, Müsteri Oltulu, & Yildiz, 2012; Chatila et al., 2014; Conickx et al., 2017; Donaldson et al., 2013; Ezzie et al., 2012; O'Leary et al., 2016; Pottelberge et al., 2011; Sato et al., 2010; Shen et al., 2017; Soeda et al., 2013). For example, the expression of let-7c and miR-125b was found to be decreased in COPD patients compared with healthy subjects. Interestingly, the expression of let-7c target genes such as tumor necrosis factor receptor type II (*TNFRII*) was found to be increased in the sputum of smoking patients with COPD, which was inversely correlated with the expression of let-7c (Pottelberge et al., 2011). In addition, miR-145 negatively regulates the release of pro-inflammatory cytokines from airway smooth muscle cells in COPD patients by targeting SMAD3, an important downstream mediator of the transforming growth factor (TGF)-β pathway (O'Leary et al., 2016). Please read (Osei et al., 2015) for an extensive review on dysregulated miRNAs in COPD and their putative implication in disease pathogenesis.

Strikingly, miRNAs do not only regulate normal gene expression but are more and more implicated in controlling epigenetic mechanisms. Along this line, the influence of miRNAs on epigenetics has been extensively studied in past years and it is well established that there is a complex interplay between miRNAs with the classical epigenetic machinery such as DNA methylation and or/histone modification. On one hand, the temporal and spatial expression of miRNA is tightly controlled by epigenetic mechanisms, such as DNA methylation of promoter regions (Xiao et al., 2015), or histone deacetylation (Zhang et al., 2012) and methylation (Vrba et al., 2010). On the other hand, miRNAs can also influence epigenetics by regulating the expression of single components of the epigenetic machinery such as histone deacetylases or DNA methyltransferases. Both interactions have been implicated in many complex human diseases as extensively reviewed elsewhere (Poddar, Kesharwani, & Datta, 2017). Future studies should address this complex interplay between miRNAs and epigenetic mechanisms, as therapeutic targeting of one component will most likely also have an effect on the other and vice versa.

Additionally, it is now well accepted that miRNAs do not only act in one particular cell type, but are actively transported within extracellular vesicles (EV). EVs are a family of information-transmitting nanovesicles, which are secreted by virtually all cell types into the extracellular space and thus can be isolated from nearly all body fluids (Mulcahy, Pink, & Carter, 2014). They are divided into different subclasses according to size and cellular origin: Exosomes are small nanovesicles (< 100 nm) derived from multivesicular bodies in late endosomes, while larger microvesicles (100 nm − 1 μm) are shed from the plasma membrane. EVs contain a large variety of bioactive molecules such as proteins, lipids, carbohydrates, and nucleic acids including small, non-coding RNAs, which can be functionally transfered upon uptake by target cells (Mittelbrunn et al., 2011). Thereby, the uptake of EVs is at least in part selectively determined by surface proteins (Mulcahy et al., 2014). Besides their function in cell homeostasis and regular cell-cell communication, EVs might also be involved in epigenetic regulatory mechanisms (Barry, 2013; Cossetti et al., 2014; Sharma, 2014; Smythies, Edelstein, & Ramachandran, 2014), as they have been shown to contain components of the epigenetic machinery, such as HDACs and DNMTs (Beckler et al., 2013; Skogberg et al., 2013). EVs from embryonic stem cells actively reprogram committed progenitors back to pluripotency (Ratajczak et al., 2006). Thereby the content of EVs differs in many diseases - which indicates a specific sorting of e.g. miRNAs into the vesicles upon injury or cellular stress, refer to (Mateescu et al., 2017) for a comprehensive review. Secretion of EVs can also be induced by exposure to cigarette smoke, mainly from bronchial epithelial cells in the lung (Kadota et al., 2016). These EVs have been shown to be enriched in pathogenic factors such as the extracellular matrix - associated protein CCN1 (Moon et al., 2014) but also miR-210 (Fujita et al., 2015). The latter has been shown to suppress autophagy, promote myofibroblast differentiation and therewith induce pathological airway remodeling in COPD (Fujita et al., 2015). Further effects on COPD pathogenesis have been described for EVs secreted from macrophages (Cordazzo et al., 2014; Li, Yu, Williams, & Liu, 2010; Zeitvogel et al., 2012) and mainly microvesicles derived from endothelial cells (Chironi et al., 2009; Heiss et al., 2008; Thomashow et al., 2013).

In summary, cigarette smoking and COPD alter the expression, and most likely also the secretion, of miRNAs into EVs. While it is well established that these mechanisms contribute to the development of airway remodeling and other COPD pathologies, it is intriguing to speculate that a differential miRNA sorting into EVs might even be implicated in systemic distribution of disease risk, i.e. also to the germline. To our knowledge this has not been investigated in COPD so far. Thus, EV-mediated transport of small RNAs could be a novel mechanism through which environmental influences on single cells in the lung could lead to systemic distribution of altered gene expression or epigenetic marks and future studies should aim to investigate these underlying mechanisms in detail.

3. Epigenetic editing

Epigenetic editing is an approach to target epigenetic effector domains to any desired gene to rewrite epigenetic marks at a defined locus (de Groote, Verschure, & Rots, 2012). Many reviews already describe the status of the field demonstrating that rewriting epigenetic marks modulates gene expression and that even silenced genes in the heterochromatin context can be re-expressed using these tools (Cano-Rodriguez & Rots, 2016; Ecker & Beck, 2017; Falahi, Sgro, & Blancafort, 2015; Sander & Joung, 2014; Tost, 2016). The designed molecular method mainly includes two constitutes: a DNA targeting system which can recognize and bind to the specific sequence in the desired site directly, and an epigenetic effector domain, which is able to alter the epigenetic state of the target locus. Up until now, various DNA binding systems and epigenetic modifiers have been applied to activate or repress gene expression directly and different transfer methods employed to introduce these agents to the cells (see Fig. 1).

3.1. DNA targeting systems

The DNA targeting systems are designed to bind to the gene of interest with at least nine, but preferably 16 (mathematically unique in the human genome) base pairs. The DNA targeting platform acts as a guide to trace its effector domain. Nowadays, three kinds of DNA binding proteins are widely used. Zinc finger proteins (ZFPs), the first engineered DNA targeting system, were the first to be employed to regulate the expression of desired genes (de Groote et al., 2014; Gommans et al., 2007; Huisman et al., 2013; van der Gun et al., 2013). These early research efforts made use of ZFPs fused to non-catalytic domains, such as VP16 (a viral transcriptional activator) and its tetramer VP64 or KRAB (a transcriptional repressor) (Magnenat & Blancafort, 2004; Beltran, Sun, Lizardi, & Blancafort, 2008; Beltran et al., 2011). Later, another programmable gene targeting protein platform, the Transcription-Activator-Like Effectors (TALEs), were introduced. However, both ZFPs and TALEs are cumbersome and resource-consuming to construct, as each newly engineered DNA-binding protein needs to be fused to the effector domain of interest. Recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-Associated Protein 9 (Cas9) system was

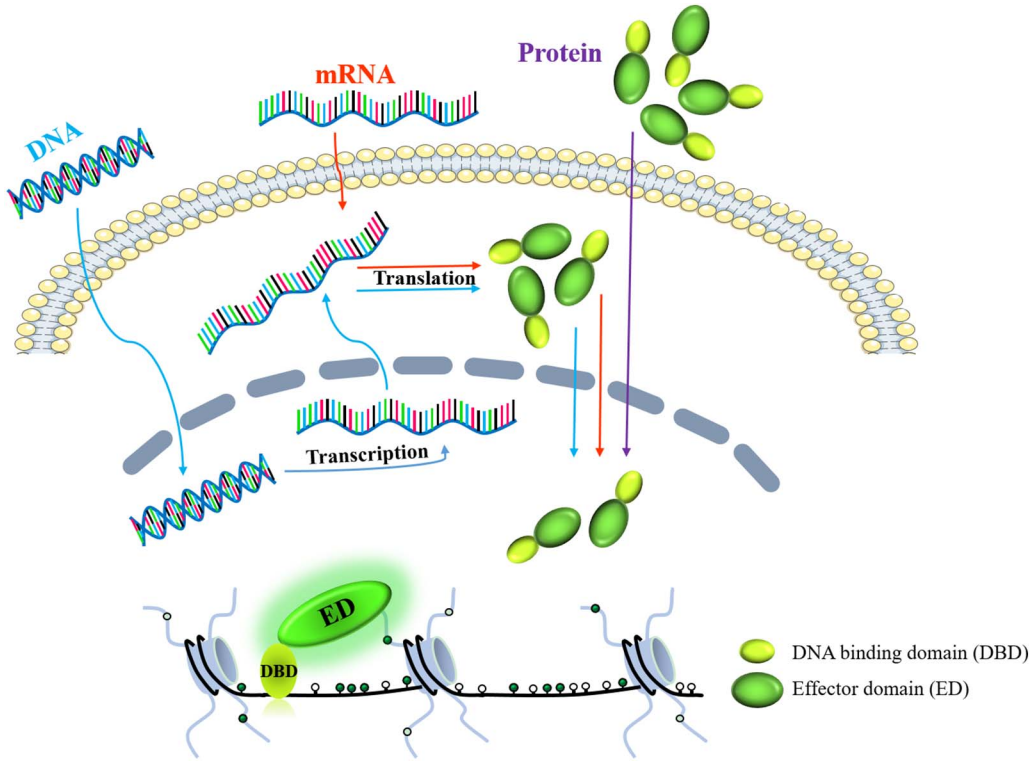

Fig. 1. Transference of various DNA binding systems and epigenetic modifiers.
Epigenetic editing is employed to rewrite epigenetic marks at the target gene of interest. The designed molecular tool mainly includes two constitutes: a DNA binding domain (DBD) which can recognize and bind to the specific sequence in the desired site directly, and an epigenetic effector domain (ED), which is able to alter the epigenetic state of the target locus. The manipulation of protein is achieved indirectly by DNA or mRNA level or directly through protein deliver, where different delivery methods can be employed.

introduced to target genes of interest in a much more straightforward manner, which made epigenetic editing available to the broader research community. CRISPR/Cas9 system comprises a single guide RNA (sgRNA) and the Cas9 protein (Hsu, Lander, & Zhang, 2014), allowing flexibility in targeting as one dCas9 fusion can be easily targeted to any gene of interest by only redesigning the sgRNA instead of constructing new fusion proteins. These DNA targeting platforms, although mainly exploited for genome engineering, make it experimentally possible to reprogram gene expression.

3.1.1. ZFPs

C2H2 type zinc finger proteins (ZFPs) are the first naturally occurring examples of predictable DNA recognition modules detected in eukaryotes and are part of numerous natural transcription factors (Wolfe, And, & Pabo, 2000). Zinc finger (ZF) arrays consist of tandem repeated ZF elements in which each finger is composed of about 30 amino acids containing one α-helix and two β-sheets, developing a coherent platform stabilized by zinc ions. Each ZF unit contains three to four important amino acids on the surface of the α-helix, which can be engineered to recognize a specific DNA triplet (Choo & Klug, 1994). Stitching engineered ZF domains together results in the recognition of longer stretches of DNA, which subsequently increase the specificity of ZFPs (Gersbach, Gaj, & Iii, 2014). ZF arrays with six or more ZF motifs stitched together can be employed to recognize 18 base pairs which ~~is~~ are a mathematically unique address in the human genome. This way, ZFPs have been explored as a powerful tool to target DNA sequence in the genome. The first constructed ZFP was engineered to repress the fusion oncogene BCR-ABL (Choo, Sánchezgarcía, & Klug, 1994). Many engineered ZFPs fused with transcriptional activators or repressors (artificial transcriptional factors) have subsequently been reported to regulate the expression of endogenous genes (de Groote et al., 2012). The main advantages of the ZFPs are the relatively small size and low immunogenicity (Falahi et al., 2015; Mussolino et al., 2011), although they are time-consuming and expensive to generate and, so far, not highly specific. Nevertheless, the first proof of concept studies on epigenetic editing are reported using engineered ZFP-fusions (Chen et al., 2014; Falahi et al., 2013; Gregory, Mikhaylova, & Fedulov, 2012; Rivenbark et al., 2012; Siddique et al., 2013; Snowden et al., 2003).

3.1.2. TALEs

Another DNA binding system, the Transcription-Activator-Like Effectors (TALEs), is derived from plant pathogenic bacteria (Boch & Bonas, 2010). TALEs consist of tandemly repeated and highly conserved 34 amino acid sections. The recognition specificity of TALEs correlates with amino acids located at 12th and 13th (Gaj & Gersbach, 2013; Sun & Zhao, 2013), a region called the repeat variable di-residues (RVD). Each RVD distinguishes a single base pair within the DNA-binding site (HD = C, NI = A, NG = T, NN = G), providing gene targeting. Similar to ZFPs, TALEs modulars are tied together to bind with adjacent DNA sites. Also TALEs have been fused to epigenetic editors to successfully modulate transcription through binding to the specific sites of the host cell genome (Konermann et al., 2013; Maeder et al., 2013). The disadvantages of TALE arrays are the extensive identical repeat sequences hampering construction and immunogenicity.

3.1.3. CRISPR-Cas9

The newest breakthrough genome targeting platform is the CRISPR system, which has revolutionized molecular biology research (Sander & Joung, 2014). The CRISPR system was first identified as the bacterial defense system, and categorized into three different types. The type II CRISPR is the simplest design, mainly consists of two components: sgRNAs and the protein Cas9 (Hsu et al., 2014). The sgRNAs function~~s~~ in the recognition of a target gene sequence of about 20 bps upstream of a 5′-NGG-3′ PAM (protospacer adjacent motif) site, which is essential for binding and cleavage. The nuclease protein Cas9, guided by a sgRNA to a specific sequence, cleaves double-stranded DNA resulting in breaks in the invading foreign gene. To regulate gene expression without altering the DNA sequence, a catalytically deactivated Cas9 (dead Cas 9, dCas9) has been engineered, which still can bind the specific genomic site but is not able to cleave it. This homing device can then be connected with transcriptional or epigenetic modulators (Sander & Joung, 2014). The advantage of the CRISPR/dCas system is

that the sgRNA is easy to design and cheap, and the nuclease-dead dCas9 molecule can be fused to different epigenetic modulators, which only need to be constructed once but can then be used for all targets. These properties make the CRISPR/Cas9 system be the most promising and widely used gene targeting platform so far.

3.2. Applications in COPD

Until now, limited reports are available regarding the application of DNA targeting systems in COPD. The airway mucus hypersecretion is one of the main contributors to the pathogenesis of COPD. One of the master regulators of mucus production is SAM-pointed domain-containing Ets-like factor (SPDEF), and silencing its expression likely reduces mucus secretion. Recently, we have shown that *SPDEF* was hypomethylated in bronchial epithelial cells after growth at an air-liquid-interface (ALI) for 2 weeks, when compared to cells from non-COPD controls. This was accompanied by increased mRNA expression of *SPDEF* and the downstream mucus-related genes *MUC5AC* and *AGR2* (Song, Heijink, et al., 2017). Furthermore, we reported that targeted silencing of *SPDEF*, using ZFPs and CRISPR/dCas platforms, successfully attenuated mucus-related gene expression, further reducing mucus production in lung epithelial cells (Song, Cano-Rodriquez, et al., 2017). This indicates that epigenetic downregulation of *SPDEF* has the potential to generate a stable effect of mucus reduction, which may be a new therapeutic approach to eventually cure patients with excessive mucus secretion.

Besides this example, DNA targeting systems have been engineered and tested in various primary cells. For example, an artificial transcriptional activator, ZFPs fused to VP64, was designed to bind the γ-globin gene promoter, to activate the expression of the developmentally silenced fetal γ-globin in primary human adult erythroblasts (Wilber et al., 2010). Moreover, designed TALEs fused to DNMT3A or TET1 were constructed to target the promoter region of Ascl1 in neural stem cells, which altered the methylation state and modulated gene expression (Lo, Choudhury, Irudayaraj, & Zhou, 2017).

4. Delivery and therapeutic potential of Epigenetic Editing

Programmable epigenetic modification with designed DNA targeting platforms could potentiate targeted therapy by treating the specific histone and DNA methylation marks that contribute to the progression of diseases. A primary challenge, however, is the development of safe and efficient delivery methods in the lung (see Fig. 2).

4.1. Barriers to lung delivery

Theoretically, epigenetic modification of gene expression in the lung could be accomplished by delivering the epigenetic editor to the epithelial surface via aerosol, the pulmonary arterial or bronchial arterial systems, or via the pleural surface. Several barriers, however, have been identified which hamper successful delivery to the lung (Kim, Duncan, Hanes, & Suk, 2016). First, the lung presents several physical and immune barriers to keep pathogens and particulates out. This includes the mucociliary clearance system, tight junctions between epithelial cells, and alveolar macrophages which will rapidly take up delivery vehicles, preventing access to the diseased cells. In addition, excessive mucus production and inflammation, which are common in asthma and COPD, will further exacerbate this challenge to successful delivery. For targeted delivery, the delivery vehicle must reach and bind its *bona fide* receptor in the polarized epithelial cells, while avoiding binding to the abundant low-affinity receptors present on the mucus glycocalyx. Additionally, delivery vectors may be inactivated by the adaptive immune responses, including both neutralizing antibodies and cytotoxic T lymphocytes, particularly after repeated administration. Especially for the viral vectors, pre-existing immunity of the host to viral vectors can also act as a barrier to efficient vector delivery. Tackling these barriers has led to the development of a range of gene/protein transfer systems.

4.2. Gene transfer with viral and non-viral vectors

Gene transfer has been widely explored in the lung and based on a number of studies, viral vectors tend to be more efficient than a non-viral vector. Especially, modified viruses with airway cell tropisms have shown some extent of success, even in the clinical setting (Sondhi, Stiles, De, & Crystal, 2017).

4.2.1. Retroviral vectors

Retroviruses, including lentiviruses, are RNA-based, and can be converted to double stranded DNA in host cells, which can then be integrated into random sites of the host genome. Genomic integration could be beneficial because of persistent expression of the transgene, but could also be a risk factor due to potential genotoxicity. Moreover, retroviruses infect actively proliferating cells only and have therefore limited application for *in vivo* lung gene therapy, as in the lung, the vast majority of cells has been terminally differentiated and the rate of proliferating cells is low. This problem has been overcome in part by lentiviral vectors which are capable of transfecting post-mitotic cells (Naldini et al., 1996). The absence of suitable cellular receptors on the apical surfaces of lung epithelial cells represents another factor hampering *in vivo* lung gene therapy. This problem can be partly overcome by co-administration of reagents which transiently open tight junctions (Leoni et al., 2015). Alternatively, pseudotyped (lentivial) vectors, such as those modified by the addition of novel surface proteins which are originated from lung tropism viruses (eg. Influenza, Sendai virus) have resulted in effective transfections. Indeed, a first-in-man lentivirus trial in patients is under preparation, where a lentiviral vector (simian immunodeficiency virus, SIV) pseudotyped with the SeV envelope proteins F and HN is being tested to fight cystic fibrosis (Alton et al., 2017). The researchers first showed that F/HN-pseudotyped SIV vector induced lifelong transgene expression in mice (both in the lungs as well as in the noses) and repeated administration led to a cumulative dose-related increase in gene expression without toxicity or loss of efficacy (Mitomo et al., 2010). Further, they found that the efficacy, toxicity and integration site profile supports further progression towards clinical trial and pre-existing and acquired immune responses did not interfere with vector efficacy (Alton et al., 2017). Therefore, these recombinant lentiviral vectors are promising for the treatment of chronic lung disease, while further development of these vectors is also necessary, including large-scale production and purification, and integrated-defective but efficient vectors.

4.2.2. Adenoviral vectors

Recombinant adenovirus (rAd) vectors, the first effective *in vivo* gene delivery vectors, are double-stranded DNA-based viruses which have natural tropism for the respiratory tract (Nemerow & Stewart, 2016). rAd vectors are capable of infecting both proliferating and non-proliferating cells, do not integrate into the host genome, and result in highly efficient gene transfer *in vivo*. rAd vectors can accommodate larger inserts (up to 8 kb) than adeno-associated viral vectors (up to 5 kb), mediate transient but high levels of protein expression, and can be easily produced at high titers. However, adenoviruses may cause high immunogenity *in vivo* because of the capsid, double-stranded DNA genome, or viral proteins expressed from the vector backbone (Nayak & Herzog, 2010). Moreover, the basolateral localization of receptors for Ad vectors limit the effectiveness of Ad vectors in lung disease therapy. “Helper-dependent” Ad was designed to reduce cellular immunity and to increase the cloning capacity transgene size through removing many of the viral coding sequences, which resulted in some successes (Józkowicz & Dulak, 2005; Nayak & Herzog, 2010; Piccolo & Brunetti-Pierri, 2014; Suzuki et al., 2013). However, at the same time, these vectors raise a safety risk issue as production and

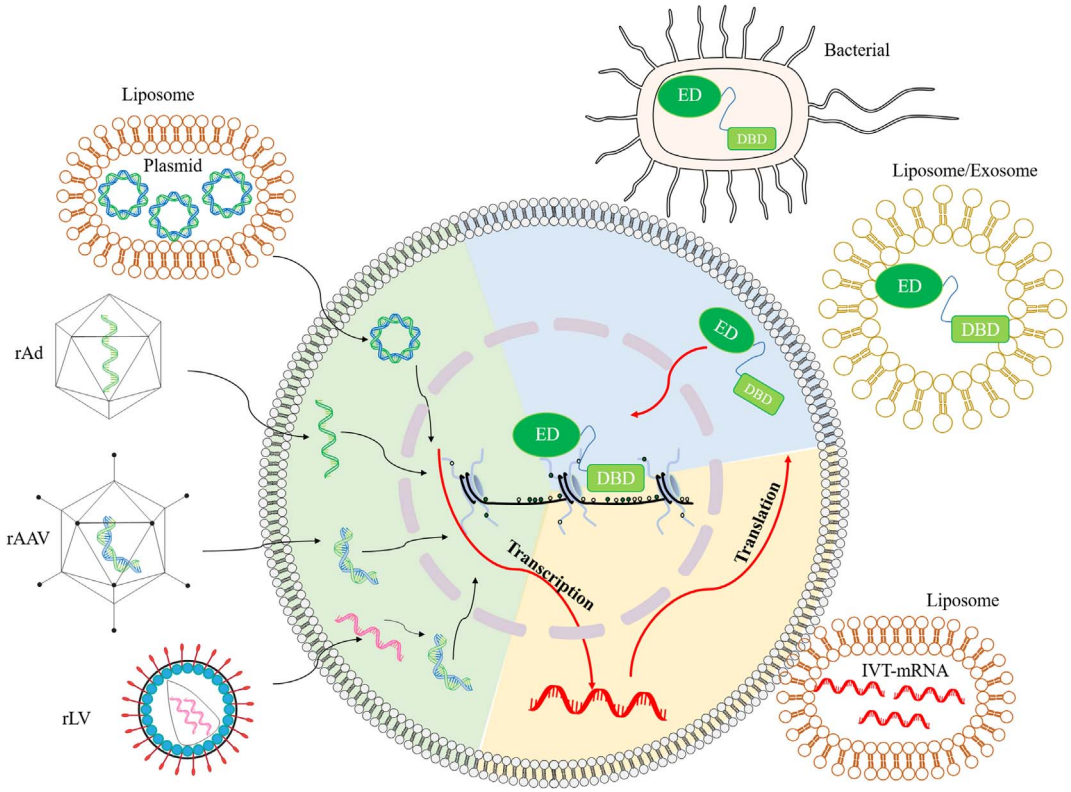

Fig. 2. Different systems to deliver epigenetic editors.
rAd = recombinant adenovirus, rAAV = recombinant adeno-associated virus, rLV = recombinant lentiviruses, IVT-mRNA = *in vitro* transcribed resulting in messenger RNA, DBD = DNA binding domain, ED = epigenetic effector domain.

contamination of replication competent Ad viruses might occur. Therefore, the Ad vector is a great platform for *in vitro* study, but currently not the preferred choice for lung gene therapy.

4.2.3. Adeno-associated viral vectors

Recombinant adeno-associated virus (rAAV) vectors are DNA viruses which have not been associated with any human disease. As such, they have received much attention as gene therapy vectors. However, AAV is also replication deficient itself, so production and replication of AAV requires help from genes of helper viruses, including Ad and herpes simplex virus (HSV). rAAV vectors are capable of transducing non-dividing quiescent cells and mediate long-term gene expression in a wide variety of tissues, including skeletal muscle, liver, central nervous system and retina (Daya & Berns, 2008). Numerous (> 100) AAV serotypes (natural or created) have been tested with diverse tissue tropism, which also help to bypass the pre-existing neutralizing antibodies in the human population. Several AAV serotypes have been shown to mediate efficient transduction of airway and alveolar epithelium. AAV delivery of ZFP- and TALE-based transcriptional regulators has demonstrated promising preclinical results in experimental animal models of Huntington's and Parkinson's disease. The recent development of smaller Cas9 systems that are compatible with AAV is a major advance in the development of CRISPR-Cas9-based gene therapy. In addition, there is a range of promising approaches to overcome the limitation imposed by the packaging capacity of AAV such as the use of dual-vector approaches (Chamberlain, Riyad, & Weber, 2016). Hung and coworkers successfully made genomic modification in mouse retinal cells with dual AAV-mediated CRISPR/Cas system *in vivo* (Hung et al., 2016). So, AAV compatible CRISPR/Cas systems have the potential to accelerate clinical applications of (*epi*)genome engineering, including in lung diseases.

4.2.4. Non-viral gene transfer

Non-viral (synthetic) vectors have been developed to avoid the use of viruses, thus minimizing the risk of immunogenicity and increasing the chance of effective repeated administration. In addition, non-viral vectors overcome the inherent shortcoming of viral vectors, such as potential genome insertional risks (retrovirus and lentivirus), limited size of the expression cassette (AAV) and the risk of creating replication competent viruses (Helper-dependent Ad, AAV). In general, however, non-viral vectors tend to be less efficient in gene transfer *in vivo* because of the paucity of specific components required for cell entry. Typically, in non-viral vectors, the therapeutic gene is cloned in plasmid DNA (pDNA) from bacteria or *in vitro* transcribed resulting in messenger RNA (IVT-mRNA), and complexed with cationic lipids or/and polymers to enhance cell entry. Non-viral vectors have been developed for delivery of pDNA and short interfering RNA. In addition, this platform has been largely adopted to delivery of IVT-mRNA due to its attractive feature, which is the fast onset of protein expression without the need to enter the nucleus in order to be functional. IVT-mRNA has inherent shortcomings because of its instability and immunogenicity, which have been substantially overcome by structural modifications.

Cationic lipids have been the most explored as non-viral vectors for pDNA and IVT-mRNA transfection so far. They can complex with pDNA or IVT-mRNA through electrostatic interaction and form lipoplexes (Rezaee, Oskuee, Nassirli, & Malaekeh-Nikouei, 2016). Strategies to improve transfection efficiency of lipoplexes have focused on improving cellular uptake and endosomal escape via optimization of lipid formulation (such as shielding the liposome surface with hydrophilic uncharged polymers polyethylene glycol (PEG)) and incorporating targeting ligand(s), which also help to reduce nonspecific binding and increase the circulation half-life *in vivo*. Lipofectamine is a good example of a commercially available liposomal formulation, which has good transfection profiles for both pDNA and IVT-mRNA but has limited utility *in vivo*, partially because of its cellular toxicity. GL67A is

another promising lipid-based formulation, which is considered as the gold standard in non-viral respiratory gene transfer because of its therapeutic potential, low toxicity and safety profile in many pre-clinical and clinical trials (Alton et al., 2015; McLachlan et al., 2011). Lipid nanoparticles (LNP) are a class of lipid-like materials. Highly effective LNPs are composed of a cationic lipid (complexes negatively charged DNA and RNA and enhances endosomal escape), a naturally-occurring phospholipid (support lipid bilayer structure), cholesterol (aids in stability), and lipid PEG derivative (decreases aggregation and nonspecific uptake) (Kanasty, Dorkin, Vegas, & Anderson, 2013). Miller and coworkers described the development of the first non-viral delivery system for *in vitro* and *in vivo* co-delivery of Cas9 mRNA and targeted sgRNA from a single LNP (Miller et al., 2017), providing powerful tools for *in vivo* gene editing and even epigenetic editing.

Cationic polymers can complex with negatively charged nucleic acids through charge-charge interaction, forming polyplexes which normally show higher stability than lipoplexes (Lachelt & Wagner, 2015). Two well-known polymer based carriers are naturally derived polymer chitosan and synthetic polymer polyethyleneimine (PEI). Chitosan is considered a biologically safe polymer for controlled delivery of DNA, siRNA and IVT-mRNA, in particular to affect local or systemic delivery of drugs via the body's mucosal barriers because of its muco-adhesive properties (Mao, Sun, & Kissel, 2010; Okamoto et al., 2003; Singh et al., 2012). PEI is also commonly used for *in vitro* and *in vivo* lung gene transfer (Davies et al., 2008; Gautam, Densmore, Golunski, Xu, & Waldrep, 2001; McLachlan et al., 2011). However, there is also evidence showing that inhaled chitosan microparticles had significant proinflammatory effects on lung tissue (Huang, Vieira, Huang, Yeh, & Chiang, 2005) and PEI can induce widespread immune cell infiltration. Mastorakos and coworkers formulated highly stable DNA nanoparticles based on biodegradable polymers, poly (β-amino esters) (PBAEs), possessing a dense coating of PEG, which helps the DNA nanoparticles to efficiently penetrate the mucus gel layer above the airway epithelium (Mastorakos et al., 2015). Importantly, these PBAE-based mucus-penetrating DNA nanoparticles (PBAE-MPPs) provided robust and long term (> 4 month following a single administration) transgene expression throughout the mouse lungs, superior to several gold standard gene delivery systems, including another mucus penetration polyplex PEGylated PEI/pDNA (Suk et al., 2014), the conventional polyplex PEGylated PLL/pDNA (Boylan et al., 2012; Konstan et al., 2004), and a lipoplex GL67A/pDNA (Alton et al., 2015). Furthermore, the transfection efficiency of PBAE-MPPs was not attenuated by repeated administrations, and no signs of toxicity were reported following intratracheal administration. Additionally, Polach et al., provide an effective delivery of siRNA to the lungs of normal mice through Staramine modified with methoxypolyethylene glycol (Staramine-mPEG), revealing significant target gene knockdown (Polach et al., 2012; Sparks et al., 2012).

IVT-mRNA and pDNA can also be loaded into hybrid nanoparticles, which comprise multiple materials (lipids, polymer and peptide) in a core-shell structure for more potent transfection. Typically, nucleic acid can be condensed with the “core” (e.g. cationic polymers) or be absorbed to the surface of the “shell” (liposomes), forming lipopolyplexes (Rezaee et al., 2016). Additionally, positively charged short peptides could also be used as “core” components (Lee et al., 2015) and polymer-based materials as substitutes for “shell” components (Bhavsar & Amiji, 2008; Palama, Cortese, D'Amone, & Gigli, 2015), aiming to optimize the formulation of the hybrid nanoparticles resulting into efficient transfection with low toxicity. Mahiny and coworkers have successfully corrected surfactant-B (SP-B) deficiency in mouse lungs using intratracheal delivery of ZFP and TALE nuclease-encoding mRNA complexed with chitosan-coated nanoparticles (Mahiny et al., 2015). This finding supports the possibility of using a biodegradable carrier with modified mRNA in lung disease therapy by providing a transient pulse of protein expression, as an alternative to traditional viral vectors.

Subsequently, both the expression level and persistence in the lung can be improved by optimization of the IVT-mRNA and pDNA molecule. Removal of CG dinucleotides from the transgene and improved vector design, including careful selection of the promoter/enhancer, resulted in non-viral vectors with persistent gene expression and a minimal immune response in mouse lung models (Bazzani et al., 2016; Padegimas et al., 2012). Modification of structural elements and nucleotides of the IVT-mRNA substantially improved its intracellular stability and translational efficiency, and reduced its immunogenicity (Guan & Rosenecker, 2017).

4.3. Protein delivery

The transient nature of therapeutic protein delivery makes it an attractive method for delivery of (*epi*) genome-editing proteins, for which advantages include (a) no risk of insertional mutagenesis (viral vector or pDNA) and (b) fewer off-target events due to reduced exposure time of the (*epi*)genetic editor within the cell. However, various challenges exist preventing efficient delivery of (*epi*)genetic-editing proteins which obstructs the *in vivo* application. These challenges include the large size, structural complexity, proteolytic instability, poor membrane permeability and endosomal entrapment (inefficient release) of the proteins. To tackle these challenges, a range of strategies have emerged in the last few years, including cell-penetrating peptides, virus-like particles, supercharged proteins, nanocarriers, polymers, and nanoparticle-stabilized nanocapsules (Fu, Tang, Hardie, Farkas, & Rotello, 2014). A pulmonary protein delivery system with microencapsulated chitosan nanoparticles has proven promising for systematic disease therapy (Al-Qadi, Grenha, Carrión-Recio, Seijo, & Remuñán-López, 2012) but also for local therapy in lung diseases. Furthermore, Barbas et al. explored the intrinsic cell-penetration properties of ZFPs and further improved the cell permeability by incorporation of tandem nuclear localization signal (NLS) repeats into the ZFP backbone. Direct protein administration of the constructs in mammalian cells resulted in efficient genome modifications, with reduced off-target effects (Gaj, Guo, Kato, Sirk, & Barbas, 2012; Liu, Gaj, Wallen, & Barbas, 2015). Additionally, the cellular uptake of ZFN proteins can be promoted by transferrin receptor-mediated endocytosis, facilitating genome editing in varieties of mammalian cells (Chen et al., 2013). Direct fusion to cell-penetrating TAT peptide (Ru et al., 2013; Wadia, Stan, & Dowdy, 2004), or conjugation to cell-penetrating poly-Arg peptides (Liu, Gaj, Patterson, Sirk, & Barbas Iii, 2014) facilitated direct TALE protein delivery for genome editing. The same principle is also applicable for gene disruption by cell-penetrating peptide-mediated delivery of a Cas9 protein and guide RNA (Ramakrishna et al., 2014). The Liu and Xu group developed a potent protein delivery system for genome editing *in vitro* and *in vivo*, using bio reducible lipid nanoparticles (enhancing the endosome escape of protein) complexed with negatively supercharged Cre recombinase or Cas9:sgRNA complexes (Wang et al., 2016; Zuris et al., 2015).

For genetic editing protein delivery, multiple viral vector systems have also been explored, like integrase-deficient lentiviral vectors (IDLVs) (Cai, Bak, & Mikkelsen, 2014; Lombardo et al., 2007), non-integrating gamma-retroviral vectors (NIRVs) (Bobis-Wozowicz et al., 2014), adenoviral vectors (AdV), and adeno-associated viral vectors (AAVs) (Ellis, Hirsch, Porter, Samulski, & Porteus, 2013; Händel et al., 2012), with higher protein expression and delivery efficiency and distinct cell tropism (especially for AAVs) due to receptor-mediated transfer. Further improvement is possible when the transduction efficiency is increased and targeting specificity via modifying the vector envelope with a cell type specific ligand or pseudotyping a specific trophism glycoprotein.

Bacteria are another emerging protein delivery tool, with the advantage of being (a) easy to manipulate and adaptable to scaling up and (b) safe, because the delivery strains can be easily eliminated using simple antibiotic treatment. The type III secretion system (T3SS) of *Pseudomonas aeruginosa* is a powerful tool for direct protein delivery

into mammalian cells and has successfully been used to deliver various exogenous proteins into mammalian cells (Hauser, 2009). Recently, the Jin group employed T3SS-mediated TALEN protein delivery for introducing precise gene modification in mouse and embryonic stem cells (ESCs) and human induced pluripotent stem cells (hiPSCs) with high efficiency (Jia et al., 2015). Importantly, the T3SS system was recently reengineered within a nonpathogenic strain of *Escherichia coli* (*E. coli*) with a safer profile, which will provide a highly flexible protein delivery platform with potential for future therapeutic applications (Reeves et al., 2015). Back in 2008, a genetically modified *E. coli* was already used as a gene therapy carrier for bactofection of lung epithelial cells *in vitro* and *in vivo* (Larsen et al., 2008). Although the bactofection resulted in significantly lower gene expression than cationic lipid GL67-mediated gene transfer, it is worthwhile to continue to explore the application potential of T3SS-mediated (*epi*)genetic editor protein delivery using modified *E. coli* in lung disease therapy.

Another fascinating method for protein delivery is "iTOP", induced transduction by osmocytosis and propanebetaine, which is reported by the Geijsen group (D'Astolfo et al., 2015). iTOP allows highly efficient delivery of native proteins, independent of any transduction peptide, including both recombinant cytoplasmic and nuclear proteins, into a wide variety of primary cell types. The underlying mechanism of iTOP is an active process mediated by hyperosmolality caused by NaCl, in combination with a transduction compound (propanebetaine), triggering macropinocytotic uptake and intracellular release of extracellularly applied macromolecules. iTOP has proved to be a highly efficient and non-integrative manner for *in vitro* delivery of recombinant Cas9 protein and in-vitro-transcribed sgRNA, which opens a new therapeutic avenue for potential protein delivery of (*epi*)genetic editors in lung disease therapy.

4.4. miRNA delivery

Being master regulators of gene expression and given their potential to regulate the epigenetic machinery, targeting miRNAs has been proposed for therapy of several complex diseases (Collison, Mattes, Plank, & Foster, 2011; Lu, Munitz, & Rothenberg, 2009; Mattes, Collison, Plank, Phipps, & Foster, 2009; Polikepahad et al., 2010). Current approaches to either therapeutically silence by antagomiRs or induce distinct miRNAs by miRNA mimics is extensively reviewed in (Comer, Ba, Singer, & Gerthoffer, 2014) and will not be discussed in detail here. There are two major problems that miRNA-based therapeutic approaches face: first, as discussed earlier, single miRNAs have numerous targets throughout the genome thus global induction or silencing might result in huge side effects. Second, efficient and non-immunogenic application of small single-stranded RNA requires intensive chemical modification or complex delivery systems such as viral or non-viral lipid based delivery systems (Lin, Chen, Zhang, & Zheng, 2014; Martin & Caplen, 2007). It can be imagined that specific and efficient delivery becomes even more difficult when targeting diseased tissue, such as small airways in COPD with massive mucus production. Strategies to use artificially designed nanoparticles seem promising, however these particles still cause high hepatotoxicity due to accumulation in the liver (Volkovova et al., 2013). In order to circumvent these problems, it has recently been suggested to exploit the natural route of (miRNA) delivery to cells, the extracellular vesicles (Alvarez-Erviti et al., 2011; Koppers-Lalic, Hogenboom, Middeldorp, & Pegtel, 2013; Zitvogel et al., 1998). Due to their cellular origin and natural function, EVs have a comparable membrane structure to cells, conserving their lipid composition, fluidity, and membrane proteins and they usually carry the uptake machinery with them, which is an advantage over liposomes and increases their stability *in vivo* (Ohno, Drummen, & Kuroda, 2016). There are several encouraging studies in animal models that EVs might be a useful tool to deliver small RNAs. In a hallmark study, targeted, autologous exosomes expressing a neurotropic protein (RVG peptide) on their surface have been shown to cross the blood-brain-barrier to deliver siRNA specifically to neurons in mice (Alvarez-Erviti et al., 2011). In this study, small RNAs were loaded into EVs by electroporation, but saponin treatment, use of porphyrins and genetic manipulation of parent cells have also been described as successful means to load desired content into EVs (Ohno et al., 2016). Ohno and colleagues have successfully delivered the let-7a tumor suppressor miRNA to EGFR positive breast cancer cells via EVs expressing the GE11 ligand for EGFR, which inhibited tumor growth (Ohno et al., 2013). Additionally, one study has provided evidence for successful and functional delivery of inhibitors and mimics of miR-155 to macrophages (Momenheravi, Bala, Bukong, & Szabo, 2014).

Due to these promising results in animal models, the safety and feasibility of using EVs in human diseases has already been assessed in several phase I trials on cancer, please see (Shin-Ichiro, Drummen, & Masahiko, 2016) for a comprehensive review. Briefly summarized, clinical trials showed the feasibility of large-scale EV-production and the administration of autologous EVs did not result in severe side effects or serious toxicity. Even though these results are highly encouraging, development of EV-based therapeutics should be handled with care. There are still some uncertainties i.e. regarding the content, the role in disease development, and the regulation of loading or secretion of EVs, that are essential to be understood in detail before exploiting EVs as therapeutic shuttles. Nonetheless, in the future, EVs might be important tools to deliberately deliver epigenetic modifiers such as miRNAs to target cells and to rewrite unfortunate epigenetic marks.

4.5. Administration strategies for treatment of lung diseases

For treatment of asthma and COPD, the routes of drug administration to the lungs are through inhalation or the bloodstream. Airside delivery of therapeutics to the lungs can be achieved via nasal instillation, intratracheal aerosolization, endobronchial spray or nebulization. Airside delivery has the advantage of preferential accumulation of drugs locally in the lungs, limiting penetration of therapeutics into the blood circulation with adverse effects for other organs. The therapeutic efficacy of inhaled drugs is continuously enhanced with the development of nanotechnology approaches (Kuzmov & Minko, 2015), which also improve the sustainability of drug release (Loira-Pastoriza, Todoroff, & Vanbever, 2014). Systemic delivery of therapeutics to the pulmonary vasculature and the pulmonary epithelial cells is achieved via intravenous administration of the drug, bearing the limitation of a short half-life of drugs in the blood stream and low accumulation and retention in the lungs. Therefore, the success of blood-based treatment for COPD will require more efficient systemic delivery methods. Systemic transport of protein or ncRNAs to the lungs could be achieved by using EVs that are homed to the lung, as they are stable in the bloodstream, usually non-immunogenic, and bear the potential to specifically target to distinct populations of lung cells when using specific receptors.

5. Future perspectives

Current developments in the field of epigenetic targeting technologies bear great promise for the treatment of complex diseases without clearly defined underlying mutation. The CRISPR/dCas9 technology in particular, which guides epigenetic enzymes to defined genomic loci to change DNA methylation or histone methylation and acetylation status for potentially sustained reprogramming holds the promise to develop novel therapies for COPD to prevent progressive pulmonary deterioration. Nonetheless, several important knowledge gaps have to be addressed to fully proceed into clinical applications.

First of all, COPD is a heterogeneous disease, potentially requiring precision medicine approaches. Thus biomarkers - which could be based on epigenetic marks - need to be developed to identify which epigenetic marks will have to be edited and in what patient groups. As different epigenetic marks have a different stability, epigenome based

biomarkers would ideally give information which patients will show a sustained therapy response and who will require continuous treatment. To proceed into biomarker development, serial EWAS are urgently needed to obtain information on the kinetics and persistence of epigenetic marks in various cell types. Second, suitable delivery systems have to be advanced and routes of drug administration need to be explored. As discussed above, targeted delivery to the lung might avoid systemic side effects, but could be prevented by excessive mucus production hampering access to the cells of interest. One approach might be the systemic application of EVs, that hold great potential due to their natural origin, their capacity to be packaged with either small RNAs but also proteins and the possibility to specifically target them to certain cell types. However, the detailed targeting mechanism of single EVs needs to be further understood and suitable surface markers for a highly-specific targeting to e.g. lung cells need to be identified. Thus, third, it needs to be clarified whether or not it will be necessary to target specific cell types and if so which, and how specificity can be ensured. Fourth, so far for most epigenetic marks associated with diseases such as COPD, it is not known if they are cause or consequence to the disease pathogenesis and thus, if correction of those marks will have a therapeutic effect. It is therefore of utmost importance for future studies to identify which kind of epigenetic marks are truly relevant for COPD pathogenesis, and which type of epigenetic modifier is most suitable to be transferred, i.e. DNMTs/HDACs/miRNAs/miRNA inhibitors or mimics, to improve the disease outcome. Furthermore, even though epigenetic editing promises to stably reprogram gene expression, more research is needed to fully understand the influence of native chromatin environment. In this respect, we recently reported the impact of DNA methylation on sustained re-expression of epigenetic silenced genes (Cano-Rodriguez et al., 2016). Also for *SPDEF*, a COPD target, long-term repression could be obtained (Song, Cano-Rodriquez, et al., 2017). Future work will hopefully overcome these critical hurdles and reveal if the approach of epigenome editing fulfills its great promise for clinical applications.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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